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Participation of the phenolic hydroxyl group of Tyr-8 in the catalytic mechanism of human glutathione transferase P1-1

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The coding region of cDNA corresponding to human class Pi glutathione transferase P1-1 was amplified by the PCR, subcloned into an expression vector, pKHP1, expressed in *Escherichia coli*, and characterized. The physicochemical and catalytic properties of recombinant glutathione transferase P1-1 were indistinguishable from those of the enzyme previously isolated from human placenta. The active-site residue Tyr-8 of the wild-type enzyme was converted into Phe by means of oligonucleotide-directed mutagenesis. The mutant enzyme Y8F displayed a 300-fold decrease in specific activity, ascribable mainly to a lowered $k_{cat.}$ (or V) value. Kinetic parameters reflecting binding affinity, $S_{0.5}$ (substrate concn. giving $\frac{1}{2}V$) and I_{50} (concn. of inhibitor giving 50% remaining activity), were only moderately elevated in the mutant enzyme. These results indicate that Tyr-8 contributes primarily to catalysis as such, rather than to binding of the substrates. The dependence of $k_{cat.}/K_m$ on pH shows an optimum at pH 7.0, defined by acidic and basic ionic dissociation constants with $pK_{a1} = 6.7$ and $pK_{a2} = 7.3$ respectively. The mutant enzyme Y8F does not display the basic limb of the $k_{cat.}/K_m$ versus pH profile, but shows a monotonic increase of $k_{cat.}/K_m$ with an apparent pK_{a1} of 7.1. The results indicate that the phenolic hydroxyl group of Tyr-8 in un-ionized form, but not the phenolate of Tyr-8, contributes to catalysis by glutathione transferase P1-1.

INTRODUCTION

Prior to the publication of the structure of the pig lung class Pi glutathione transferase (GST) at 0.23 nm (2.3 Å) resolution (Reinemer *et al.*, 1991), no definitive information was available on active-site residues for any GST. Chemical modification (cf. Mannervik, 1985) and site-directed mutagenesis experiments (Stenberg *et al.*, 1991*a*) had indicated the importance of arginine residues for binding of glutathione to the G-site (Mannervik *et al.*, 1978), a function substantiated by the three-dimensional structure of the pig lung enzyme. However, no clear evidence was available for amino acid residues directly involved in the catalytic mechanism. The X-ray diffraction analysis identified a tyrosine residue (Tyr-7), which is conserved not only in the class Pi GSTs (Reinemer *et al.*, 1991), but also in the other GST classes (Mannervik *et al.*, 1985). The hydroxyl group of this tyrosine residue in the pig enzyme appears to form a hydrogen bond with the sulphonyl group of glutathione sulphonate bound to the G-site of the enzyme. It is therefore plausible that the same functional group may interact with the thiol group of the proper substrate, glutathione, and thereby facilitate its nucleophilic attack on the second, electrophilic, substrate molecule. In order to test this hypothesis, the equivalent tyrosine residue in the homologous human class Pi enzyme, GST P1-1 (for nomenclature, see Mannervik *et al.*, 1992), was mutated into phenylalanine by means of oligonucleotide-directed mutagenesis.

EXPERIMENTAL

Materials

The plasmid pGPi2 was generously supplied by Professor Masami Muramatsu, University of Tokyo, Tokyo, Japan. Radioactive nucleotides were from Amersham International (Amersham, Bucks., U.K.). The phage vector M13mp18 and sequencing reagents were obtained from Boehringer–Mannheim (Mannheim, Germany). Enzymes for DNA restriction and modification were purchased from commercial suppliers. *S*-Hexylglutathione was synthesized and immobilized to Sepharose 4B as described by Mannervik & Guthenberg (1981). Oligonucleotides were synthesized by Operon Technologies (Alameda, CA, U.S.A.) with the following sequences: GRA6, 5′-ATGGATCCTGCAGTTAGCTCTCTTAGAAATT; GRA7, 5′-GTGAATTCAGGAGCCACCATGCCGCCCTAC; HUMPIY8F, 5′-GAACTGGGAAGAAGACCACGGT.

Plasmid and phage constructions

The plasmid pGPi2 was used as a template for add-on PCR. Oligonucleotides GRA7 and GRA6 were designed to generate a functional ribosomal binding site in front of the coding sequence, as well as *Eco*RI and *Pst*I restriction sites flanking the amplified DNA. The reaction system contained 1 mM-$MgCl_2$, 200 μM-dNTPs, 0.4 μM of each of the oligonucleotides, 4 nM linearized pGPi2 plasmid and 0.5 unit of *Taq* polymerase. The reaction was run for 30 cycles on a DNA Thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). The *Eco*RI and *Pst*I restriction sites of the amplified DNA were used for insertion behind the *tac* promoter in the plasmid pKK223-3 (Pharmacia LKB Biotechnology, Uppsala, Sweden) to give pPCRPi. DNA sequencing (Sanger *et al.*, 1977) showed a single mutation in codon 198 of the coding sequence. Therefore the major part of the PCR-derived insert, between the *Bst*EII and the *Pst*I cleavage sites (codons 34–210), was replaced by DNA from the original plasmid pGPi2, to give the plasmid pKHP1.

The insert of pKHP1 was transferred into M13mp18, using *Eco*RI restriction sites, for sequencing as well as for site-directed mutagenesis (Taylor *et al.*, 1985). The Y8F mutation expected by use of the mutamer HUMPIY8F was verified by nucleotide sequencing and the GST P1-1 cDNA was then subcloned into the expression plasmid pKK-D (Björnestedt *et al.*, 1992) to give

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; V, maximum velocity; $S_{0.5}$, substrate concn. giving 0.5 V; I_{50}, concn. of inhibitor giving 50% remaining activity.

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pKHP1Y8F, which was transformed into *E. coli* JM103 for expression of the recombinant protein.

Expression and purification of recombinant proteins

E. coli JM103 containing the plasmid pKHP1 or the corresponding mutants were grown in 3 litres of RS broth [2% (w/v) tryptone, 1.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1% (v/v) glycerol and 50 mg of ampicillin/ml], equally distributed in six 2-litre Erlenmeyer flasks on a rotary shaker at 200 rev./min. At an A_{555} value of 0.35, isopropyl-β-D-thiogalactopyranoside (0.2 mM final concentration) was added to the culture medium.

The bacteria were grown for 20 h and then collected by centrifugation for 5 min at 7000 *g* and resuspended in an equal volume of 10 mM-Tris/HCl, pH 7.8, 50 mM-EDTA, 15% (w/v) glucose, and 1 mg of chicken egg white lysozyme/ml. After 1 h on ice, the cells were disrupted by sonication and centrifuged for 20 min at 25000 *g*. After addition of phenylmethanesulphonyl fluoride to 170 μM, the supernatant fraction was passed over Sephadex G-25 (Pharmacia) in a 4 cm × 40 cm column equilibrated with buffer A [10 mM-Tris/HCl, pH 7.8, 1 mM-EDTA, 0.2 mM-dithiothreitol, 0.02% (w/v) NaN_3]. Active fractions were pooled and combined with 25 g of *S*-hexylglutathione–Sepharose 4B affinity matrix (Mannervik & Guthenberg, 1981). The mixture was kept at 10 °C with gentle shaking for at least 2 h. The gel matrix was washed thoroughly on a glass filter funnel with buffer B (buffer A fortified with 0.2 M-NaCl), resuspended in buffer B, and packed on a column. The enzyme was finally eluted with buffer B supplemented with 5 mM-*S*-hexylglutathione. The eluted protein was concentrated by ultrafiltration and dialysed for 14 h against four changes of 2 litres of buffer A.

Analysis of purified proteins

Protein concentrations were determined by the method of Peterson (1977). Purity and homogeneity were checked on SDS/PAGE [12.5% (w/v) acrylamide/0.5% (w/v) *NN'*-bisacrylamide] with subsequent silver staining (Blum *et al.*, 1987). H.p.l.c. analysis (Ostlund Farrants *et al.*, 1987) was performed on a Dynamax 300 A reverse-phase C4 column (Rainin Instruments, Woburn, MA, U.S.A.). The elution system used was 1 min of 45% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid; followed by a 20 min gradient of 45–60% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (Widersten *et al.*, 1991.

RESULTS

Expression of recombinant human GST P1-1

The original expression clone, pPCRPi, obtained by PCR amplification of the coding region of pGPi2 (Kano *et al.*, 1987), produced a functional protein with full catalytic activity. However, nucleotide sequence analysis demonstrated a change in codon 198 from GAG (Glu) into GGG (Gly), and the isolated protein displayed an isoelectric point (pI 5.3) that was higher than the value for the wild-type enzyme (pI 4.6). Therefore codons 34–210 were replaced by the authentic sequence excised from pGPi2. The final construct was sequenced in its entirety on both strands and found to contain the expected wild-type sequence.

The recombinant wild-type GST P1-1 was expressed in *E. coli* JM103 in a yield of 3.5–4 mg per litre of bacterial culture medium. The yield of the E198G mutant, obtained by PCR, was not significantly different. A second mutant form of GST P1-1, Y8F, was created by mutating codon 8 from TAT (Tyr) into TTC (Phe). This mutant protein was also expressed in *E. coli* at a similar level to the wild-type enzyme.

After affinity purification on immobilized *S*-hexylglutathione, the recombinant enzymes were homogeneous as judged by reverse-phase h.p.l.c., SDS/PAGE and isoelectric focusing.

In terms of physico-chemical properties, the mutant forms Y8F and E198G were indistinguishable from the wild-type enzyme, with the exception of the deviant isoelectric point of mutant E198G. Specifically, the apparent subunit molecular mass (22 kDa) and the retention time in the h.p.l.c. system were not significantly different from those of the wild-type enzyme. The elution position of the symmetrical peak obtained after gel filtration on Superose 12 (Pharmacia FPLC System) corresponded to an apparent molecular mass of 40 kDa, demonstrating that both mutant forms and wild-type enzyme were dimers.

Kinetic studies

The Y8F mutant of human GST P1-1 displayed a drastically reduced catalytic activity as compared with the wild-type enzyme. The activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate, and under standard conditions mutant Y8F showed only 0.3% of the wild-type specific activity (Table 1). Rate saturation curves at pH 6.5 were determined using fixed high concentrations of each of the substrates glutathione and CDNB at various concentrations of the other substrate. At the fixed concentration of glutathione (6 mM), saturation was achieved with respect to this substrate, whereas at the highest concentration of CDNB attainable (2 mM, limited by the solubility), the reaction rate had not reached its limiting value. GSTs do not obey strictly the Michaelis–Menten equation (Mannervik & Danielson, 1988) but the maximum velocity, V, and the substrate concentration giving half-maximal velocity, $S_{0.5}$, could be obtained by curve-fitting (Bardsley *et al.*,

Table 1. Kinetic parameters and inhibition characteristics of recombinant GST P1-1 and mutant Y8F

Measurements were made in 100 mM-sodium phosphate (pH 6.5) and 2% (v/v) ethanol at 30 °C. Specific activity was measured with 1 mM-glutathione (GSH) and CDNB. Complete plots of v versus varied [S] with constant [GSH] (6 mM) or constant [CDNB] (2 mM) respectively were obtained. V is the maximal velocity obtained by extrapolation, and $S_{0.5}$ is the substrate concentration giving $\frac{1}{2}V$. The $k_{cat.}/K_m$ was calculated from the slope of the graph at a low concentration of the varied substrate (Danielson & Mannervik, 1985). I_{50} is the concentration of inhibitor giving 50% remaining activity with 1 mM-CDNB and 1 mM-GSH. The specific activity values are given as means ± S.D. as determined from replicates (n = 5). Other parameter values ± S.D. were obtained by regression analysis. Values in parentheses indicate the mutant value as a percentage of the wild-type value.

Parameter (unit)	Wild-type	Mutant Y8F
Specific activity (μmol·min^{-1}·mg^{-1})	128 ± 4	0.43 ± 0.01 (0.34)
Varied [GSH]		
V (μmol·min^{-1}·mg^{-1})	287 ± 40	5.3 ± 0.1 (1.9)
$S_{0.5}$ (mM)	0.5 ± 0.1	1.9 ± 0.1 (380)
$k_{cat.}/K_m$ (mM^{-1}·s^{-1})	345 ± 9	1.18 ± 0.04 (0.34)
Varied [CDNB]		
V (μmol·min^{-1}·mg^{-1})	570 ± 30	11 ± 2 (1.9)
$S_{0.5}$ (mM)	2.6 ± 0.2	3.1 ± 0.7 (120)
$k_{cat.}/K_m$ (mM^{-1}·s^{-1})	83 ± 2	1.48 ± 0.04 (1.8)
Inhibition characteristics		
I_{50}, *S*-hexylglutathione (μM)	25 ± 10	52 ± 20 (200)
I_{50}, *S*-*o*-iodobenzylglutathione (μM)	3.3 ± 0.8	33 ± 13 (1000)

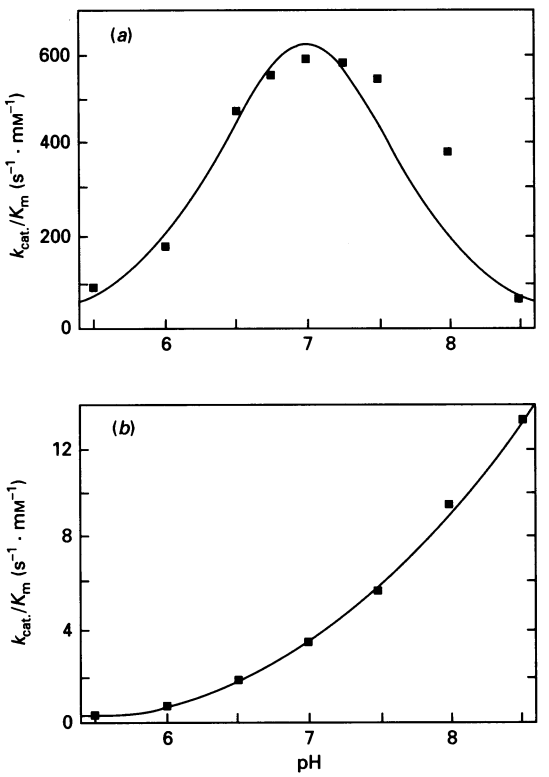

Fig 1. pH-dependence of the $k_{cat.}/K_m$ of wild-type and Y8F mutant human GST P1-1

(*a*) Wild-type and (*b*) Y8F mutant parameters were determined at 30 °C using a low concentration of glutathione and fixed high concentration of CDNB (1 mM) in 0.1 M-sodium phosphate at the pH values indicated. The curves were fitted by eqns. (1) and (2) using the SIMFIT program (Bardsley *et al.*, 1989).

1989). As an estimate of $k_{cat.}/K_m$, the initial linear part of the v versus [S] curve was used, for which $v = (k_{cat.}/K_m) \cdot [E]_{tot.} \cdot [S]$ (Danielson & Mannervik, 1985), where $[E]_{tot.}$ is the subunit concentration based on a molecular mass of 23.4 kDa.

Table 1 shows that, for mutant Y8F, the $k_{cat.}/K_m$ values for glutathione and CDNB were approx. 0.3% and 1.8% respectively of the values for the wild-type enzyme. The corresponding V and $S_{0.5}$ values suggest that the major effect of the mutation is a decreased turnover number ($k_{cat.}$ or V), but that a decreased apparent affinity for the substrate also contributes. Another measure of affinity for active-site ligands was obtained by determination of I_{50} (concn. of inhibitor giving 50% remaining activity) for inhibitory glutathione derivatives (Table 1). *S*-Hexylglutathione demonstrated a 2-fold and *S*-*o*-iodobenzylglutathione a 10-fold loss of affinity, as indicated by the I_{50} values. These changes are of the same magnitude as the changes in $S_{0.5}$ values.

The pH-dependence of $k_{cat.}/K_m$ was determined for the wild-type enzyme as well as for the mutant Y8F (Fig. 1). The wild-type enzyme demonstrated a clear pH optimum at pH 7.0, and the curve was fitted by the simplest equation that would display an optimum (Tipton & Dixon, 1979):

$$k_{cat.}/K_m = \frac{K_3}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} \quad (1)$$

with $K_{a1} = 10^{-6.7}$ M and $K_{a2} = 10^{-7.3}$ M (K_3 is a parameter determining the amplitude). In contrast, the pH profile for mutant Y8F demonstrated a continuous increase of $k_{cat.}/K_m$ as a function of pH, which could be fitted with:

$$k_{cat.}/K_m = \frac{K_3}{1 + \frac{\{H^+\}}{K_{a1}}} \quad (2)$$

where $K_{a1} = 10^{-7.1}$ M.

DISCUSSION

In order to make possible structure–activity studies of human GST P1-1, heterologous expression of the recombinant enzyme in *E. coli* was performed. Thus site-directed mutagenesis of DNA encoding the protein was used for investigation of defined structural components of the enzyme. The initial attempts to subclone the relevant DNA segment from the original GST P1-1 clone, pGPi2 (Kano *et al.*, 1987), were based on the available restriction sites and partial digestion with exonuclease *Bal*31 (M. Widersten, M. Ericsson & B. Mannervik, unpublished work). However, no product suitable for expression of the wild-type enzyme was obtained. Neither were attempts at generating an *Nco*I restriction site by a site-specific mutation at the 5′ end of the coding region successful (R. H. Kolm & B. Mannervik, unpublished work). Some of the problems associated with the construction of the expression clone of GST P1-1 appeared to be caused by formation of secondary structure in the 5′ region of the DNA. Therefore the coding region of the plasmid pGPi2 was copied and amplified by means of PCR using primers that would introduce suitable restriction sites as well as configuration with respect to the Shine–Dalgarno box of the expression vector that would be favourable for translation of the corresponding mRNA.

The recombinant protein obtained from the plasmid pKHP1 was indistinguishable from the wild-type enzyme purified from human placenta (Guthenberg & Mannervik, 1981). Further, the coding nucleotide sequence was checked and found to be identical with that of the original clone. By use of a suitable oligonucleotide, HUMPIY8F, a mutation in codon 8 was generated and the mutant protein Y8F could be produced. Excepting the kinetic properties, this mutant protein was essentially indistinguishable from the wild-type enzyme.

The mutant Y8F of GST P1-1 differs from the wild-type enzyme by having the phenolic hydroxyl group of Tyr-8 replaced by a hydrogen atom. The homologous wild-type class Pi GST from pig lung shows 82% positional identities with the human enzyme at the amino acid level, and must therefore (Chothia & Lesk, 1986) have a chain fold very similar to that of its human counterpart. Thus it can safely be concluded that Tyr-8 is a component of the human enzyme active site in a similar manner as in the pig enzyme (Reinemer *et al.*, 1991). Examination of the known primary structures of cytosolic GSTs shows that the Tyr residue is conserved in all structures, indicating an important role in the enzymes. On the basis of this conservation in evolution, the corresponding Tyr residue close to the *N*-terminus in human class Alpha GST A1-1 has previously been subjected to mutagenesis (Stenberg *et al.*, 1991*b*). The results of that study strongly suggested that Tyr-8 in GST A1-1 contributes to catalysis, even though it is not strictly required.

The results of the present investigation demonstrate that removal of the hydroxyl group of Tyr-8 causes an approx. 300-fold decrease in specific activity (Table 1), an effect approx. 10-fold greater than that observed with GST A1-1 (Stenberg *et al.*, 1991*b*). For both enzymes the kinetic consequences of the tyrosine mutation are expressed predominantly in $k_{cat.}/K_m$ and V values (Table 1 and Stenberg *et al.*, 1991*b*). Kinetic parameters reflecting binding, i.e. $S_{0.5}$ and I_{50}, were only affected to a small degree in both enzymes. This finding indicates that the role of the conserved

tyrosine residue is primarily linked to catalysis as such, rather than to substrate binding.

The dependence of $k_{cat.}/K_m$ on pH in the wild-type GST P1-1 may as a first approximation be considered to be governed by two protonic equilibria characterized by apparent pK_a values of 6.7 and 7.3 (Fig. 1). The lower value corresponds to the ascending limb of the pH profile, whereas the higher value represents the descending limb. Assuming that only two ionizing groups are involved, the results imply that the basic form of the conjugate acid–base pair with a pK_{a1} of 6.7 and the acid form of the second conjugate base–acid pair with a pK_{a2} of 7.3 are essential for activity. The ionizations involve groups on the enzyme as such, since they are expressed in the $k_{cat.}/K_m$ values and since the substrates used do not have any pK_a values near pH 7 (Cleland, 1982).

In contrast to the wild-type enzyme, mutant Y8F appears to display only the ascending limb of the $k_{cat.}/K_m$ versus pH curve. The apparent pK_{a1} value of 7.1 is similar to the corresponding value (pK_a 6.7) for the wild-type enzyme. The most important difference is that the basic limb of the wild-type pH profile is totally absent in the mutant profile (Fig. 1). Since the only structural difference between the two enzymes is the hydroxyl group of Tyr-8, this finding suggests that ionization of this phenolic hydroxyl group is linked to the decrease in $k_{cat.}/K_m$ at higher pH values. Fitting of eqn. (1) gave an apparent pK_{a2} value of 7.3 for this ionization, but the slight discrepancy between the predicted and the experimental values in this region suggest that more than one ionization is involved and that the true pK_a value of Tyr-8 may be somewhat higher.

The main conclusion from the pH-dependence of $k_{cat.}/K_m$ is that the hydroxyl group of Tyr-8 has to be protonated in order to be active in catalysis. This excludes the possibility that Tyr-8 in an ionized form may serve as a base facilitating ionization of glutathione, which is believed to be a crucial step in the catalytic mechanism of the GSTs (Jakoby & Habig, 1980; Mannervik & Danielson, 1988; Graminski *et al.*, 1989). A more plausible alternative based on the present findings is that the hydroxyl group of Tyr-8 activates the sulphur of the glutathione molecule by hydrogen bonding (Stenberg *et al.*, 1991*b*).

The nature of the acidic group ionizing with a pK_{a1} of 6.7 is unclear. However, the three-dimensional structure of the pig enzyme (Reinemer *et al.*, 1991) shows that neither histidine nor cysteine residues occur in the active site. A carboxyl group, such as that of the conserved Asp-96 contributed by the neighbouring subunit, is a possible candidate that could serve as proton acceptor in the catalytic mechanism. However, the nature of this residue has to be established by additional mutations of the protein.

In conclusion, the present investigation indicates that the phenolic hydroxyl group of Tyr-8 in un-ionized form contributes to the catalytic process of GSTs by hydrogen bonding. The elucidation of the full catalytic mechanism will depend on further studies based on relationships between structure and activity.

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